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Trypsin-catalysed formation of pig des-(23–63)-proinsulin from desoctapeptide-(B23–30)-insulin

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Incubation of pig desoctapeptide-(B23–30)-insulin with trypsin in solvent systems consisting of dimethyl sulphoxide, butane-1,4-diol and Tris buffer resulted in the formation of an extra peptide bond between Arg-B22 and Gly-A1 in the DOPI molecule. This DOPI derivative can also be regarded as pig des-(23–63)-proinsulin. The structure of the new, previously unreported, proinsulin analogue was determined on the basis of amino acid analysis, dansylation and digestion with *Staphylococcus aureus* V8 proteinase. Receptor-binding ability of des-(23–63)-proinsulin was 20% of that of pig desoctapeptide-(B23–30)-insulin and 0.02% of that of pig insulin.

INTRODUCTION

Desoctapeptide-(B23–30)-insulin (DOPI) is an important substrate for the enzyme-assisted semisynthesis of insulin and insulin analogues by the method of Inouye *et al.* (1979, 1981*a*). In most of the cases reported so far, DOPI was *N*-protected before trypsin-catalysed couplings (Inouye *et al.*, 1979, 1981*a*, *b*; Gattner *et al.*, 1980; Tager *et al.*, 1980; Cao *et al.*, 1981; Cui *et al.*, 1983; Shoelson *et al.*, 1983; Kobayashi *et al.*, 1984*a*, *b*).

We have successfully used unprotected DOPI as a substrate for trypsin-catalysed semisyntheses (T. Kubiak & D. Cowburn, unpublished work), and observed an unusual by-product under some conditions. This product is shown here to be pig des-(23–63)-proinsulin (DproI).

MATERIALS AND METHODS

Materials and general methods

Zinc pig insulin (lot 7NR38B) was purchased from the Elanco Product Co. (Indianapolis, IN, U.S.A.). Trypsin [L-1-chloro-4-phenyl-3-tosylamidobutan-2-one ('TPCK')-treated] was obtained from Worthington Biochemical Corp. Sephadex G-50 (fine grade) was from Pharmacia. Tris was from Sigma Chemical Co., and silica-gel plates HLF from Analtech. Other reagents were of analytical grade from Aldrich Chemical Co. and were used without further purification. The pH values reported are the apparent values, direct readings obtained with a glass electrode, uncorrected for the presence of organic solvents.

Hydrolyses (6 M-HCl) were carried out in evacuated Pierce hydrolysis tubes at 110 °C for 24 h in the presence of phenol (1 drop per 1 ml of 6 M-HCl) unless otherwise specified. Amino acid analyses were performed on a Spinco model MS amino acid analyser.

Receptor-binding assay was conducted with liver plasma membranes (McCaleb & Donner, 1981).

DOPI was prepared from zinc-free insulin (Insulin Research Group, Shanghai Institute of Biochemistry, 1976) as described by Tager *et al.* (1980). The crude product was purified on Sephadex G-50 in 3 M-acetic acid.

DOPI incubation with trypsin: illustrative procedure

A sample of DOPI (20 mg, 4 μmol) was dissolved in 0.72 ml of the solvent system dimethyl sulphoxide/butane-1,4-diol/0.25 M-Tris/acetate buffer (pH 7.5) (5:5:4, by vol.) and the pH was adjusted to 7.00 at 25 °C with 3 M-acetic acid (0.024 ml). Then trypsin powder (2 mg) was added. This dissolved rapidly and the mixture was incubated at 37 °C for 4 h. The DOPI concentration was 5.4 mM, and the water content of the buffer and acetic acid was 28% (v/v). The enzyme action was stopped by the addition of 50% (v/v) acetic acid (0.8 ml), and the mixture was loaded on to a Sephadex G-50 column (1 cm × 90 cm), which was eluted with 3 M-acetic acid at a flow rate of 24 ml/h; fractions (1.6 ml each) were monitored at 280 nm. Fractions corresponding to the 5000-M_r material were pooled and freeze-dried. The yield was 15.2 mg. The products were isolated from the 5000-M_r material by either preparative h.p.l.c. on Zorbax ODS (Fig. 1) or on DEAE-Sephadex A-25 (Cui *et al.*, 1983) as shown in Fig. 2(*a*).

Characterization of the unknown

Trypsin digestion of the unknown (0.5 mg) was conducted in 0.2 ml of 0.1 M-*N*-ethylmorpholine buffer, pH 8, containing EDTA (2 mM), $CaCl_2$ (20 mM) and trypsin (0.05 mg). The mixture was incubated for 3 h at 37 °C. After 2 h and 3 h small portions were taken, diluted with h.p.l.c. solvent A and examined by h.p.l.c. on the Zorbax ODS column. The experiment was accompanied by a control that lacked trypsin.

Performic acid-oxidized (Hirs, 1967) unknown was danylated and hydrolysed as described by Gray (1972).

Digestion with *Staphylococcus aureus* V8 proteinase was performed by the method of Chance *et al.* (1981). After digestion the mixture was frozen, freeze-dried, then redissolved in h.p.l.c. solvent A (0.3 ml) and separated by preparative h.p.l.c. on Zorbax C-8 (Fig. 4*a*). Material corresponding to peak 1A was collected, freeze-dried and purified by preparative paper chromatography on Whatman 3MM paper (6 cm × 12 cm) in propan-2-ol/1 M-acetic acid (2:1, v/v). The band containing

Abbreviations used: DOPI, desoctapeptide-(B23–30)-insulin; DproI, des-(23–63)-proinsulin; Dns or dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

Sakaguchi-positive (Greenstein & Winitz, 1961) and chlorine/tolidine-positive material (R_F 0.7) was cut out and eluted with 3 M-acetic acid. The eluate was concentrated under reduced pressure, filtered through a Pasteur pipette containing a plug of glass-fibre paper and freeze-dried. Half of this material was hydrolysed (6 M-HCl, 110 °C, 48 h) and subjected to amino acid analysis. One-fifth of the remainder was dansylated in the medium containing *N*-ethylmorpholine (Gray, 1972). The *N*-terminal arginine was identified in the 6 M-HCl hydrolysate of the dansylated sample by comparison with standard Dns-arginine on electrophoresis in 8% (v/v) formic acid (Gray, 1972). The same procedures were used for the isolation and characterization of the (A1–4)-pentapeptide from the DOPI digest (Fig. 4*b*, peak AB material), as well as the (A13–17)-pentapeptide fragment (Fig. 4, peaks 2A and 2B).

RESULTS AND DISCUSSION

In model studies on the use of trypsin as a catalyst for peptide-bond formation the optimum pH for the enzyme-catalysed syntheses was found to be near 6.5 (Inouye *et al.*, 1979). The presence of organic solvents, such as dimethylformamide or dimethyl sulphoxide, or polyhydroxy alcohols in the reaction medium greatly shifted the peptide-bond equilibrium towards synthesis. Inouye *et al.* (1979, 1981*a*, *b*) successfully pioneered the use of semisynthetic procedures involving trypsin-assisted coupling of the t-butoxycarbonyl-protected DOPI with synthetic (B23–30)-octapeptide(s) for the preparation of human insulin and a variety of insulin analogues.

When unprotected DOPI was incubated with trypsin under similar conditions to those used by Inouye *et al.* (1981*a*) (Table 1, Expt. 1), h.p.l.c. on Zorbax ODS revealed the presence of an extra peak at R_t 26 min, observed along with that of DOPI at R_t 11.6 min (Fig. 1). The same unknown product was present along with the DOPI in the ~ 5000-M_r material after Sephadex G-50 chromatography. Formation of the unknown was accompanied by significant oligomerization, since only 42% of the ~ 5000 M_r material was recovered after gel filtration. The rest of it was converted by the enzyme into higher-M_r products, which were co-eluted with trypsin (Table 1, Expt. 1). Neither the unknown nor oligomerization was observed when DOPI was incubated in the reaction medium without trypsin (Table1, Expt. 2). Other conditions used for the DOPI incubation with trypsin are listed in Table 1.

Oligomerization was concentration- and pH-dependent. Generally, at lower initial DOPI concentrations (approx. 5 mM) less of the higher-M_r products was found (Table 1, Expts. 3–7). In the pH range 6.4–6.7 relatively more oligomers were formed as compared with similar experiments conducted at pH 6.0 or 7.0 (Table 1, Expts. 1, 3 and 4). The formation of the unknown depended on pH and the butane-1,4-diol content in the incubation mixtures as shown in Table 1. It was observed that the reaction reached its equilibrium after 4 h of incubation. A prolonged incubation time led to more oligomers,

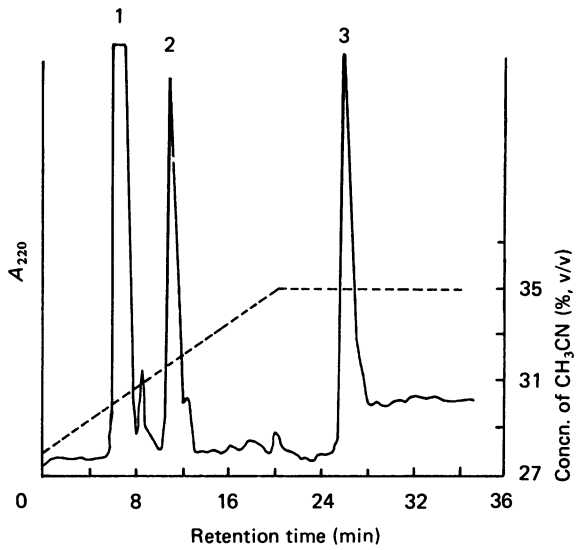

Fig. 1. H.p.l.c. profile of the DOPI/trypsin incubation mixture on a DuPont Zorbax ODS column (0.94 cm × 25 cm)

Solvent A consisted of 0.125 M-NaH_2PO_4 adjusted to pH 2.45 with H_3PO_4. A 20 min linear gradient from 28% to 35% (v/v) acetonitrile in solvent A was used. The final solvent composition was maintained for an additional 15 min. The flow rate was 2 ml/min, with monitoring at 220 nm. Peaks: 1, solvent peak; 2, DOPI; 3, DproI.

Table 1. DOPI incubation with trypsin

The water content of the incubation mixture includes 0.25 M-Tris/acetate buffer and the diluted acetic acid used to adjust the pH. The 5000-M_r material was obtained by chromatography on Sephadex G-50, and the DOPI and DproI contents were determined by the h.p.l.c. peak heights on Zorbax ODS.

Expt. no.	Initial concn. of DOPI (mM)	Me_2SO/1,4-Bu(OH)$_2$ ratio (v/v)	Water content (%, v/v)	pH	DOPI/trypsin ratio (w/w)	Incubation time (h)	5000-M_r material (%)	DOPI content in 5000-M_r material (%)	DproI content in 5000-M_r material (%)
1	10.0	1:1	24	6.7	10:1	19	42	49	51
2	10.0	1:1	24	6.7	10:0	19	91	100	0
3	4.3	1:1	32	6.4	10:1	19	61	68	32
4	4.2	1:1	32	6.4	10:1	4	65	69	31
5	5.4	1:1	28	7.0	10:1	4	76	77	23
6	5.2	1:2	28	7.0	10:1	4	80	56	44
7	5.2	1:2	29	6.0	10:1	4	89	82	18

Table 2. Amino acid compositions of DOPI and related peptides (theoretical values in parentheses)

Peptides 1A, 1B, 2A and 2B were isolated by h.p.l.c. (Fig. 4) followed by preparative paper chromatography. A hydrolysis time of 40 h was used for peptides 1A and 1B.

	Amino acid composition (mol of residue/mol)							
Amino acid	DOPI	Dns–DOPI	DproI	Dns–DproI	Peptide 1A	Peptide 1B	Peptide 2A	Peptide 2B
Asp	2.97 (3)	3.15 (3)	3.22	3.3	0.25	0.40		
Thr	1.09 (1)	1.08 (1)	0.93	1.06	0.05	0.04		
Ser	2.63 (3)	3.02 (3)	2.89	2.93	0.17	0.30		
Glu	7.0 (7)	6.79 (7)	7.00	6.77	0.92 (1)	1.2 (1)	1.98 (2)	1.96 (2)
Gly	3.02 (3)	2.35 (2)	3.48	3.33	1.00 (1)	1.00 (1)		
Ala	1.2 (1)	1.20 (1)	1.28	1.30	0.10	0.10		
Cys*	+	+	+	+	−	−	−	−
Val	2.6† (4)	3.01† (4)	2.83†	2.97	1.12 (1)	0.80† (1)		
Ile	1.1† (2)	1.23† (2)	1.07†	1.30	1.07 (1)	0.86† (1)		
Leu	6.33 (6)	6.05 (6)	5.81	6.49			2.00 (2)	1.97 (2)
Tyr	3.26 (3)	0‡	3.14	0‡	0.14	0.20	0.98 (1)	1.01 (1)
Phe	0.91 (1)	0.32 (0)	0.99	0.32				
His	2.32 (2)	2.10 (2)	1.95	2.02				
Arg	1.19 (1)	0.99 (1)	1.02	1.06	1.01 (1)			

* Half-cystine values were not integrated.
† Low values due to incomplete hydrolysis under the conditions used.
‡ Tyrosine was converted into *O*-Dns-tyrosine.

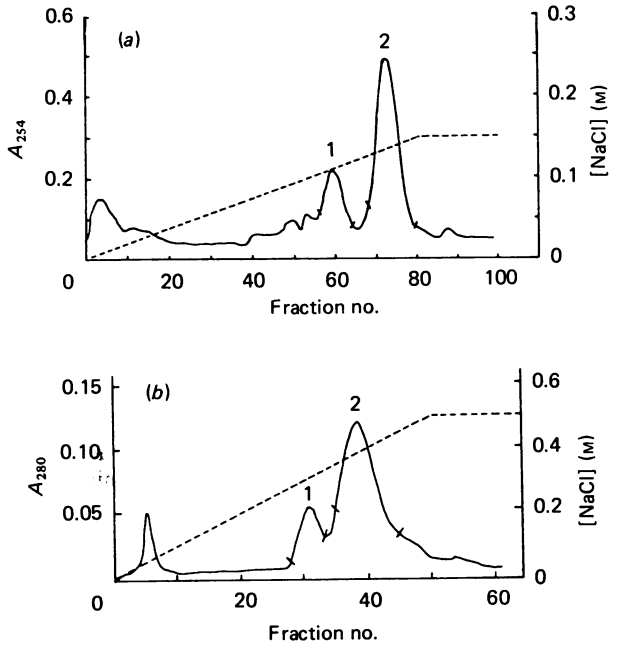

Fig. 2. Ion-exchange chromatography of the mixture of DproI and DOPI

(*a*) The Sephadex G-50-purified incubation mixture (5000-M_r material, 37 mg) was dissolved in the initial buffer (15 ml) and loaded on to a DEAE-Sephadex A-25 column (1 cm × 20 cm). The initial buffer was 40% propan-2-ol in 0.05 M-Tris adjusted to pH 7.6 with HCl (200 ml), and the final buffer was 0.15 M-NaCl in the initial buffer (200 ml); a combination of linear gradient and isocratic elution was used (see the gradient line ------). The fraction size was 5 ml. Pools 1 and 2 were concentrated under vacuum and desalted on Sephadex G-10. Peaks: 1, DproI (8.7 mg); 2, DOPI (25.5 mg). (*b*) SP (sulphopropyl)-Sephadex C-25 chromatography of the mixture of DOPI and DproI. The column dimensions were 2 cm × 25 cm. The initial solvent was 3 M-acetic acid (100 ml), and the final solvent was 3 M-acetic acid containing 0.5 M-NaCl (100 ml); a linear gradient of 0–0.5 M-NaCl was used (------). The fraction size was 4 ml. Pools 1 and 2 were desalted on Sephadex G-10. Peaks: 1, DproI (1.1 mg); 2, DOPI (5.5 mg).

though the ratio of DOPI to the unknown remained essentially the same.

Enzyme-catalysed formation of the unknown was reversible. Trypsin hydrolysed the unknown to DOPI in aqueous 0.1 M-*N*-ethylmorpholine buffer, pH 8, when no organic solvents were added. Within a digestion period of 2 h the h.p.l.c. peak of the unknown disappeared while that of DOPI emerged.

The amino acid composition of the unknown was found to be the same as that of DOPI (Table 2). However, the unknown was not a DOPI dimer, judged by its Sephadex G-50 elution volume.

The unknown was also generated from DOPI by trypsin in the solvent system consisting of dimethyl sulphoxide and Tris/acetate buffer with no butane-1,4-diol added. Therefore the possibility of ester formation between butane-1,4-diol and the Arg-B22 residue was excluded.

Since the unknown was more hydrophobic than DOPI, on the basis of its h.p.l.c. retention time, we considered the case of trypsin-catalysed acylation of the hydroxy groups of tyrosine, serine and/or threonine by the Arg-B22 residue in DOPI. *O*-Acyl derivatives of the three amino acids mentioned above are known to be readily hydrolysed under mild conditions to generate the original functional groups (Bodanszky *et al.*, 1976). The hydrolysis proceeds more rapidly in the presence of ammonia or amines, and is complete within minutes (Bodanszky *et al.*, 1976; Riordan & Vallee, 1972). No DOPI was formed from the unknown on incubation with 2.8% (v/v) ammonia or aq. 50% (v/v) isopropylamine

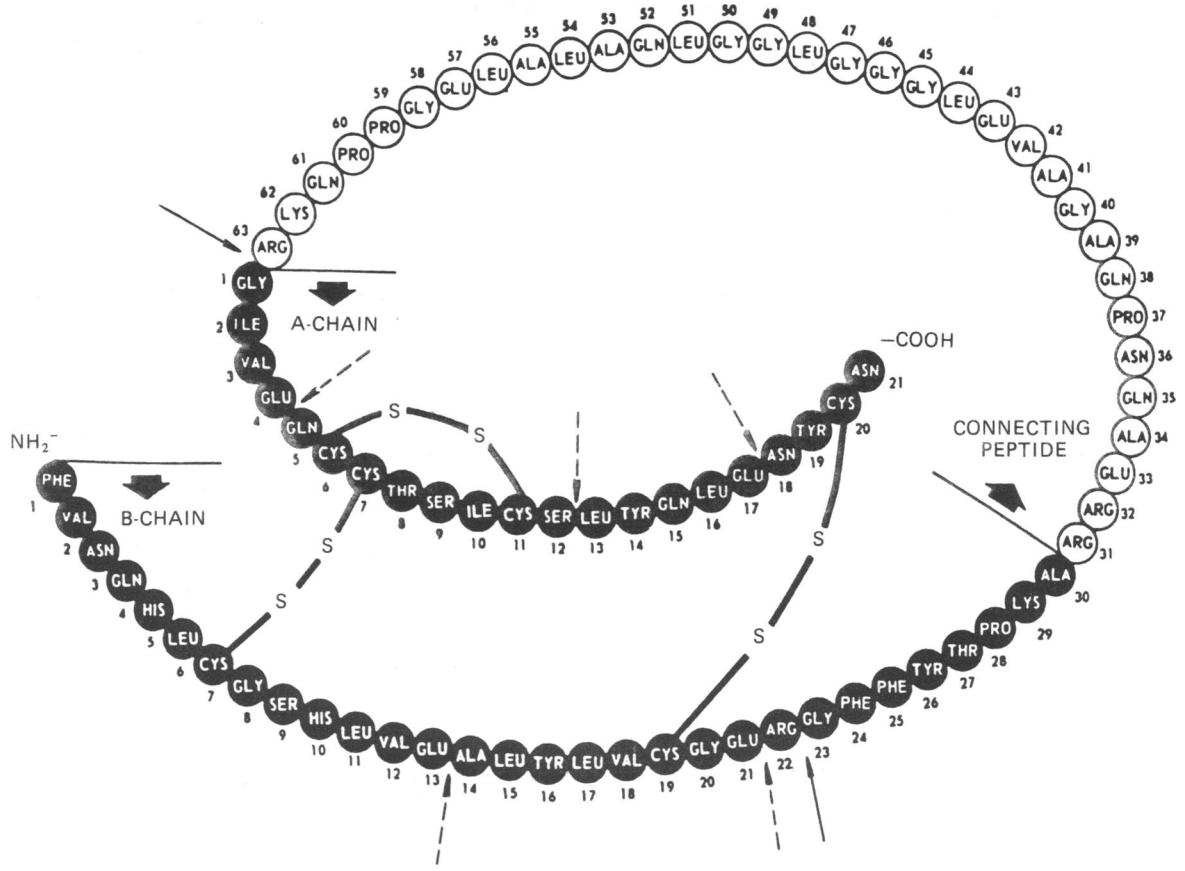

Fig. 3. Primary structure of pig proinsulin (Show & Chance, 1968)

The sequence of pig insulin is represented by the amino acids in dark circles. Thin unbroken arrows indicate the sequence that is deleted in DproI. Broken arrows show expected *S. aureus* proteinase cleavage sites in DOPI.

within a period of 4 h. Therefore it was concluded that the unknown does not possess any ester bonds.

The unknown was eluted before DOPI from a DEAE-Sephadex column (Fig. 2*a*), as well as from an SP-Sephadex column (Fig. 2*b*), which indicated that it has at least one less negative and one less positive charge as compared with DOPI under the conditions used for separations.

Dansylation, performed on the performic acid-oxidized unknown, followed by 6 M-HCl hydrolysis, revealed only the presence of the *N*-terminal phenylalanine residue. It turned out that the Gly-A1 was blocked and not available for dansylation, since all three glycine residues were found in the dansylated unknown hydrolysate as compared with only two in the hydrolysate of Dns-DOPI (Table 2).

The above results show that the trypsin-catalysed peptide-bond formation between the Arg-B22 and Gly-A1 residues took place leading to the conversion of DOPI into the unknown. This new DOPI derivative can also be regarded as a proinsulin analogue, namely DproI (Fig. 3).

To prove that such a proinsulin analogue was generated, the unknown was subjected to digestion with *S. aureus* V8 proteinase under the conditions described for insulin by Chance *et al.* (1981). *S. aureus* V8 proteinase specifically hydrolyses peptide bonds on the carboxy side of glutamic acid residues (Houmard & Drapeau, 1972). In addition, it was determined that in the insulin molecule an extra cleavage occurred at the Ser-A12–Leu-A13 bond (Chance *et al.*, 1981). If trypsin had catalysed formation of the peptide bond between the Arg-B22 and Gly-A1 residues, we should expect to find the Arg-B22-(A1–4)-pentapeptide in the *S. aureus* V8 proteinase digest of the unknown.

As reported by Chance *et al.* (1981), the (A1–4)-tetrapeptide, deriving from insulin, was eluted in the very first peak from a Zorbax C-8 column. Since the hypothetical Arg-B22-(A1–4)-pentapeptide should be more hydrophilic than the (A1–4)-tetrapeptide, we expected it to appear on h.p.l.c. in the eluate at the same retention time as, or earlier than, the (A1–4)-tetrapeptide.

The Zorbax C-8 profiles of the *S. aureus* proteinase digest of the unknown and DOPI are shown in Fig. 4. The peak 1A fraction from the digest of the unknown (Fig. 4*a*) was further analysed on t.l.c. It contained a chlorine/tolidine-positive and Sakaguchi-positive material, with R_F 0.30, different from arginine (R_F 0.12), and a minor chlorine/tolidine-positive Sakaguchi-negative contaminant, with R_F 0.8 (Table 3). The arginine-containing peptide (Sakaguchi-positive material) was isolated and desalted by preparative paper chromatography. The same procedures were used for the final purification of the Zorbax C-8 fractions 1B, 2A and 2B (Figs. 4*a* and 4*b*). It is worth noting that neither fraction

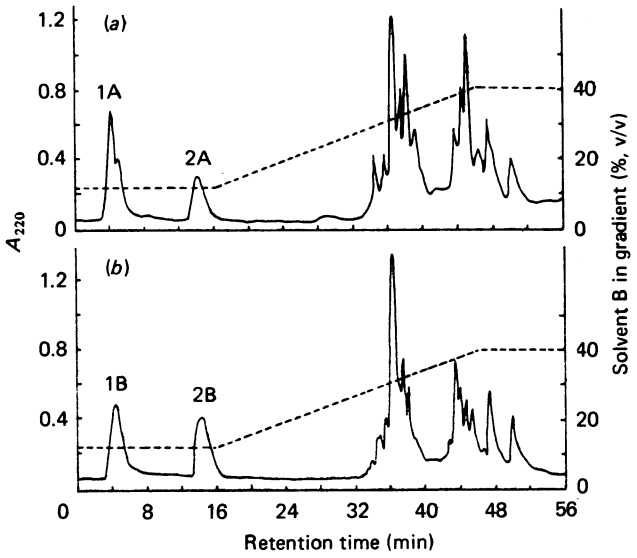

Fig. 4. H.p.l.c. profiles for the *S. aureus* proteinase digest of 3 mg of DproI (*a*) and 3 mg of DOPI (*b*)

The chromatography was accomplished with a DuPont Zorbax C-8 column (0.46 cm × 25 cm) by using slight modifications of the conditions of Chance *et al.* (1981). Solvent A consisted of 0.1 M-$(NH_4)_2HPO_4$ in water/methoxyethanol (19:1, v/v) and solvent B was acetonitrile/methoxyethanol (19:1, v/v). A combination of isocratic and gradient elution was used (see the gradient line ------). The column was thermostatically maintained at 40 °C. The flow rate was 1 ml/min. Peaks: 1A, Arg-B22-(A1–4)-pentapeptide; 1B, (A1–4)-tetrapeptide; 2A or 2B, (A13–17)-pentapeptide.

1A nor fraction 1B contained free arginine, as shown by t.l.c. (Table 3). The amino acid compositions of peptides isolated from fractions 1A, 1B, 2A and 2B (Table 2), and their *N*-terminal amino acids, determined by dansylation, were consistent with the sequences Arg-B22-(A1–4)-pentapeptide, (A1–4)-tetrapeptide, (A13–17)-pentapeptide and (A13–17)-pentapeptide respectively. Other peaks in the chromatograms of Fig. 4 show slight differences between DOPI and DproI, presumably because of incomplete digestion under the conditions used, similar to those observed by Chance *et al.* (1981).

The presence of the Arg-B22-(A1–4)-peptide in the *S. aureus* V8 proteinase digest of the unknown confirmed the trypsin-catalysed conversion of DOPI into DproI.

DproI showed 0.02% of the receptor-binding ability of that of pig insulin and 20% of that of DOPI.

Note added in proof (received 18 December 1985)

After this paper was submitted, Markussen *et al.* (1985) reported the trypsin-catalysed formation of the single-chain des-(B30)-insulin, the des-(Ala-B30) analogue with a peptide bond between Lys-B29 and Gly-A1. The maximum yield obtained was about 13%, compared with approx. 50% for DproI reported here, and this difference might possibly reflect closer location or less restricted reaction for the Arg-B22/Gly-A1 reaction in DOPI compared with the Lys-B29/Gly-A1 reaction in des-(Ala-B30)-insulin. Both des-(Ala-B30)-insulin and DproI show very low activity compared with insulin, of the order of that of proinsulin (Chance *et al.*, 1968).

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Table 3. Peptides isolated from the *S. aureus* V8 proteinase digests of DproI and DOPI

Chromatograms were developed in the solvent system propan-2-ol/1 M-acetic acid (2:1, v/v). Key: S, Sakaguchi-positive material, C, chlorine/tolidine-positive material.

	Fraction (see Fig. 4)	R_F values: T.l.c. on silica-gel plates (5 cm × 10 cm)	R_F values: Paper chromatography on Whatman 3MM (6 cm × 12 cm)
Proteinase digest of DproI	1A	0.3 (C, S); 0.8 (C)	0.7 (C, S); 0.88 (C)
	2A	0.83 (C)	0.92 (C)
Proteinase digest of DOPI	1B	0.74 (C); 0.8 (C)	0.85 (C); 0.88 (C)
	2B	0.83 (C)	0.92 (C)
Standard	Arg	0.12 (C, S)	0.32 (C, S)

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